Symbiosis, 18 (1995) 57–68
Balaban, Philadelphia/Rehovot

Bacteria Associated to Arbutoid Mycorrhizae in *Arbutus unedo* L.

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Received August 7, 1994; Accepted January 21, 1995

Abstract

Investigations on ultrastructural aspects of arbutoid mycorrhizae in *Arbutus unedo* showed the occurrence of bacteria between the hyphae of the mantle. Sequential cuts of mycorrhizal tissue revealed the spatial distribution of bacteria: in the outer region of the hyphal mantle many bacteria were present in the microniches formed by the hyphae interwoven around the roots; in the inner region bacteria formed microaggregates enclosed between hyphae, immersed in an electron-dense matrix. Quantitative and qualitative determinations showed the consistent occurrence of *Azospirillum*-like bacteria in the mycorrhizosphere and endosphere, whichever inoculum was utilized for the syntheses of arbutoid mycorrhizae. The hypothesis of a strict association between arbutoid mycorrhizae and *Azospirillum*-like bacteria is discussed.

Keywords: *Arbutus unedo*, mycorrhizae, ultrastructure, bacteria, mycorrhizosphere

1. Introduction

The occurrence of many microorganisms in mycorrhizal associations has been reported by many authors; they have been shown to possess different activities in relation to mycorrhizal fungi, in some cases behaving as antagonists (McAfee

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and Fortin, 1986; Summerbell, 1987), in other cases behaving as "helper", by promoting plant growth (Strzelczyk and Pokojska-Burdziej, 1984; Strzelczyk and Rozycki, 1985; Meyer and Linderman, 1986; Paulitz and Linderman, 1989; Garbaye and Bowen, 1989; Garbaye et al., 1990), or by fixing N_2 (Tilak et al., 1989; Li and Hung, 1987; Li et al., 1992). Moreover, N_2-fixing bacteria have been reported to be associated with ectomycorrhizal roots (Li and Hung, 1987), and N_2-fixing *Azospirillum* have been isolated from spores of arbuscular mycorrhizal fungi (Tilak et al., 1989). Recently, the diazotroph *Acetobacter diazotrophicus* was shown to infect sugarcane by means of arbuscular mycorrhizae (Paula et al., 1991).

The importance of the findings on microorganisms associated with mycorrhizae is evidenced by the current use of the term "mycorrhizosphere" (Rambelli, 1973; Linderman, 1988; 1992).

In ultrastructural studies the occurrence of bacteria has often been reported, both in mycorrhizal fungal hyphae and in plant tissues: nevertheless, they have been described as bacteria-like organisms, and no attempts have been made to isolate or classify them (Mosse, 1970; MacDonald and Chandler, 1981; Fusconi and Bonfante, 1984).

In a previous paper, the synthesis of an arbutoid mycorrhiza in *Arbutus unedo* was described (Giovannetti and Lioi, 1990). While extending the research to ultrastructural aspects of the symbiosis, during different developmental stages, bacteria, preliminarily determined as *Azospirillum* spp., were consistently found within the fungal mantle (unpublished results). Therefore, ultrastructural investigations were performed on the mycorrhizae of *A. unedo*, synthesized in the laboratory with inoculum from two diverse and distant areas, with the aim of understanding the nature of the relationship established between bacteria and *A. unedo* mycorrhizae.

2. Materials and Methods

Plant material

Seeds of *Arbutus unedo* were obtained from Florsilva Anzaloni (Bologna, Italy), and stored at 4°C until used. After surface sterilization with 30% H_2O_2 for 15 min and three rinses in sterile water, the seeds were germinated in sterile sand, in Petri dishes, at 21°C in the dark. One month later, the seedlings were transferred into the synthesis medium.

Inoculum sampling

Roots from *A. unedo* were collected, together with soil around them, from two distant and different areas in Tuscany (Italy): a) a woody hill, described in a previous paper (Giovannetti and Lioi, 1990); b) a cultivated hill, where *A. unedo* grew at the border of olive groves, near San Vincenzo (Livorno, Italy), about 100 km south of the former area.

Soil and mycorrhizal roots from the two sites were used as inoculum for the synthesis of arbutoid mycorrhizae in *A. unedo* seedlings germinated in the laboratory.

Synthesis of mycorrhizae

After germination, seedlings of *A. unedo* were transplanted into 100 ml plastic pots containing a mixture of sterile sand and unsterile experimental soil (1:1, v/v). Two experiments were set up, with 10 replicate plants for each. Plants were grown in a glasshouse with a day-time temperature of 21°C, a night-time temperature of 10°C, and a maximum light intensity of 30.7 klx. They were watered daily by an automatic device. Six- and 12-month old seedlings were harvested and checked for mycorrhiza formation under a dissecting microscope; morphologically different mycorrhizae were freed from each root system for further processing.

Transmission electron microscopy (TEM)

Root apices showing morphologically different mycorrhizae or no mycorrhiza formation, were sampled. Portions of samples were washed, fixed in 3% glutaraldehyde in 0.5 M phosphate buffer, pH 6.1, for 4 hr at 4°C, then washed several times in the same buffer, postfixed in 1% Osmium tetroxide for 3 hr at room temperature, dehydrated in a graded ethanol series and embedded in Epon-Araldite. Ultrathin sections were cut by using a diamond knife, stained with uranyl acetate followed by lead citrate, and examined under a Siemens Elmiskop 1A electron microscope.

Estimation of Azospirillum-like bacteria

For mycorrhizosphere analysis, 2 gr root apices, showing either morphologically different mycorrhizae or no mycorrhiza formation, were sampled, then transferred to 100 ml sterile distilled water (SDW), containing 5 gr glass beads and vigorously shaken for 10 min. Serial 10-fold dilutions were

then prepared and known aliquots of the suspensions were distributed in Döbereiner's nitrogen-free semi-solid agar medium, for MPN counts (Döbereiner et al., 1976).

For endosphere analysis, the same root samples used for mycorrhizosphere counts were utilized. They were thoroughly washed three times in SDW, then homogenized using a pestle and mortar; the tissue was then suspended in SDW, diluted and counted as described above.

For non-rhizosphere soil analysis, samples of soil were taken from different zones, approximately 4–6 cm away from the root system, mixed, diluted and analyzed as previously described.

3. Results

Transmission electron microscopy

Two different types of mycorrhizal roots, one arbutoid and one of the *Cenococcum*-type, were detected on *A. unedo*, regardless of the origin of the inoculum used for their synthesis.

Transmission electron microscopy analysis showed the occurrence of bacteria only in the arbutoid-type mycorrhiza. The location pattern of bacterial occurrence in 12-month old plants is described in Fig. 1. Bacteria were present both in interhyphal spaces in the fungal mantle proximal to root cells and in root cells colonized by the fungus (Fig. 1).

Figs. 2A and B show septal structures, revealing that the unknown symbiont possessed simple septa and associated Woronin bodies characteristic of ascomycetous hyphae.

Sequential cuts of 6-month old mycorrhizal tissue, from the outer to the inner part, showed the following spatial distribution: in the outer region of the hyphal mantle many bacteria were present in the interhyphal spaces (Fig. 1), showing the tendency to occupy the microniches formed by the hyphae interwoven around the root (Figs. 3A, B, C, and D); in inner regions of the hyphal mantle bacteria formed homogenous colonies between hyphae, immersed in an electron-dense matrix (Figs. 3C and D). Interestingly, the electron-dense matrix disappeared all around bacterial cells, suggesting a possible utilization of it by the bacteria (Figs. 3B and D).

The ultrastructural morphological appearance of the bacteria did not change in either young or old mycorrhizae: bacteria were morphologically identical, whereas the microniches appeared more defined, microbes occupying all the volume in the interhyphal space in mature mycorrhizae (Figs. 4A, B, C, and D). Moreover, it is important to note that the bacteria appeared

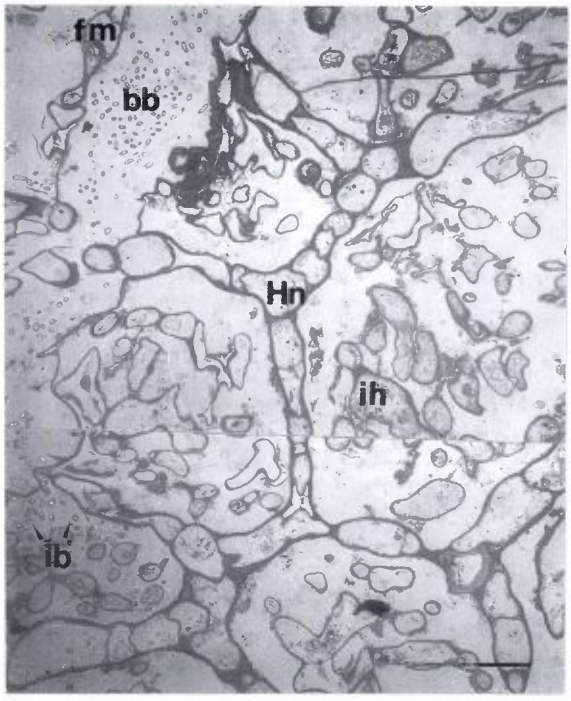

Figure 1. Transmission electron micrograph of a transverse section of a 12-month old arbutoid mycorrhiza of *Arbutus unedo*: (fm) = fungal mantle; (Hn) = Hartig net; (ih) = intracellular hyphae; (bb) = bacterial colonies between fungal mantle and first layer of epidermal root cells; (ib) = bacteria within an epidermal root cell. Bar = 10 μm.

morphologically similar, in mycorrhizae synthesized with inoculum sampled from either one of the sampling areas, which are 100 km apart.

In old senescent mycorrhizae, epidermal cells invaded by the fungus showed the occurrence of the same bacteria (Figs. 5A and B).

All the bacteria observed in the arbutoid mycorrhizae were characterized by large inclusions of lipid material (probably poly-β-hydroxybutyrate) either those within the same aggregate or colony, or those occurring in different areas. The bacteria occurring within plant cells appeared to be enclosed by a continuous membrane-like structure (Figs. 5D and E), which was not observed around bacteria external to the cells (Fig. 5C).

Extramatrical hyphae appeared to be enclosed in an electron-dense matrix, supporting the growth of many bacteria, which occurred near or attached to it (Fig. 6).

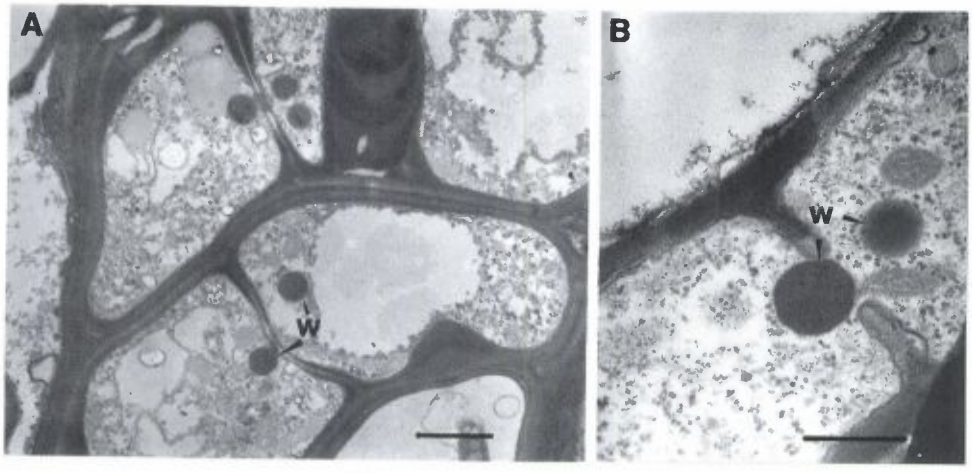

Figure 2. (A) Transmission electron micrographs of hyphae of the unknown ascomycetous fungal symbiont causing arbutoid mycorrhizae: fungal hyphae are endowed with simple septa and Woronin bodies, (B) showing an open ascomycetous septum: (w) = Woronin bodies. Bars = 1 μm.

Estimation of Azospirillum-like bacteria

The quantitative determination of *Azospirillum*-like bacteria evidenced a clear "rhizosphere effect" in the mycorrhizosphere and endosphere (Table 1). In fact, the number of *Azospirillum*-like bacteria occurring in the soil was significantly lower than that in the mycorrhizosphere and endosphere. In the "*Cenococcum*-type" mycorrhizae and in non-mycorrhizal roots the bacteria were not found.

Table 1. Numbers of *Azospirillum*-like bacteria occurring in the soil, in the mycorrhizosphere and endosphere of mycorrhizal roots of *Arbutus unedo*, synthesized with inoculum from two different areas.

Samples	Woody hill	Cultivated hill
Soil	5.2×10^2a *	1.1×10^2a
Mycorrhizosphere	2.0×10^6b	3.5×10^5b
Endosphere	6.0×10^7b	9.5×10^6b

* Values in columns followed by the same letter do not differ significantly at P = 0.01.

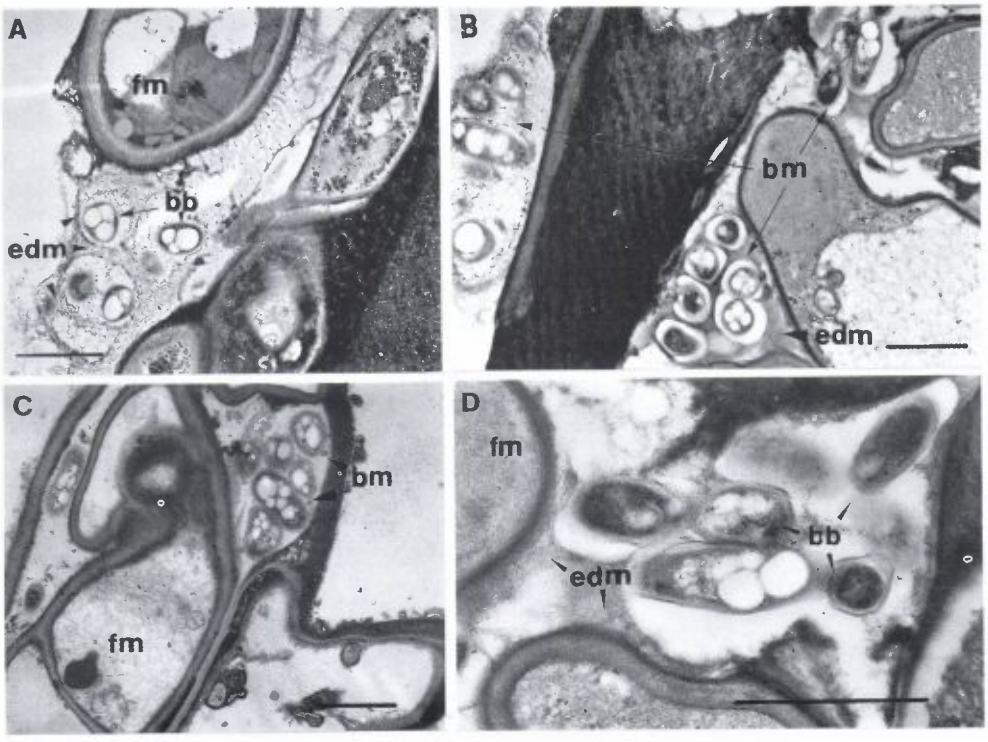

Figure 3. Transmission electron micrographs of 6-month old arbutoid mycorrhizal tissue, showing bacteria occupying the interhyphal spaces of the fungal mantle. Both outer (A) and inner regions (B, C) show homogenous bacterial cells forming microaggregates enclosed in an electron-dense matrix. This matrix disappears around bacterial cells (B, D): (fm) = fungal mantle; (bb) = bacteria; (edm) = electron-dense matrix; (bm) = bacterial microaggregates. Bars = 1μm.

Qualitative analysis, based on selectivity of the culture medium, evidenced that the bacteria probably belonged to the genus *Azospirillum*: in fact, the nitrogen-free culture medium where bacteria were inoculated, turned progressively blue from the surface to the bottom and showed a dense, white pellicule of bacterial growth 1–2 mm below the surface. The bacteria growing in this layer were short vibroid rods, 1 μm diam, had a rotating corkscrew type of mobility, and contained many inclusion granules.

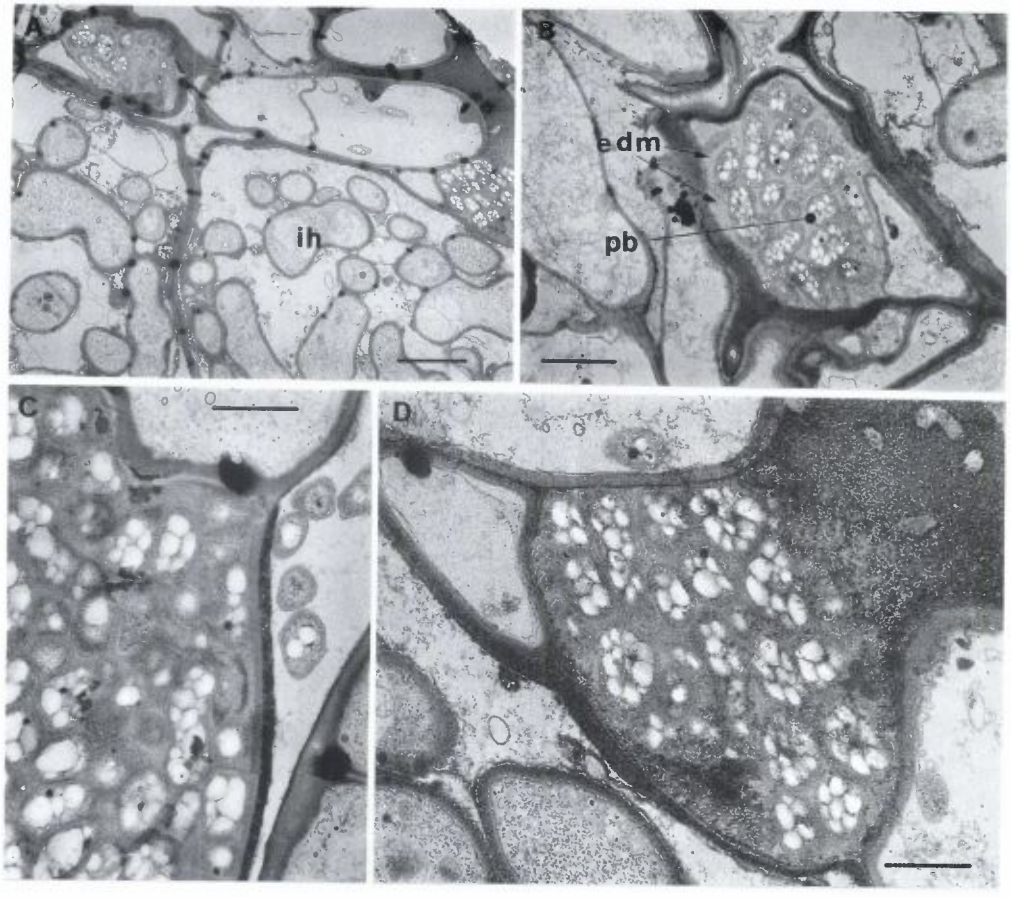

Figure 4. Transmission electron micrographs of sections of 12-month old arbutoid mycorrhizae showing pockets of bacteria between hyphae of the outer region. (A, B) general view; (C, D) showing bacterial colonization of interhyphal spaces: (ih) = intracellular invasion of fungal hyphae; (pb) = pockets of bacteria; (edm) = electron-dense matrix. Bars = 1μm.

4. Discussion

Our work shows that: i) *Azospirillum*-like bacteria occur in the hyphal mantle of the arbutoid mycorrhizae of *A. unedo;* and ii) this occurrence is not casual, because the same bacteria were consistently found in the mycorrhizosphere and endosphere of *A. unedo* arbutoid mycorrhizae, whichever inoculum had been used for their syntheses. These results suggest

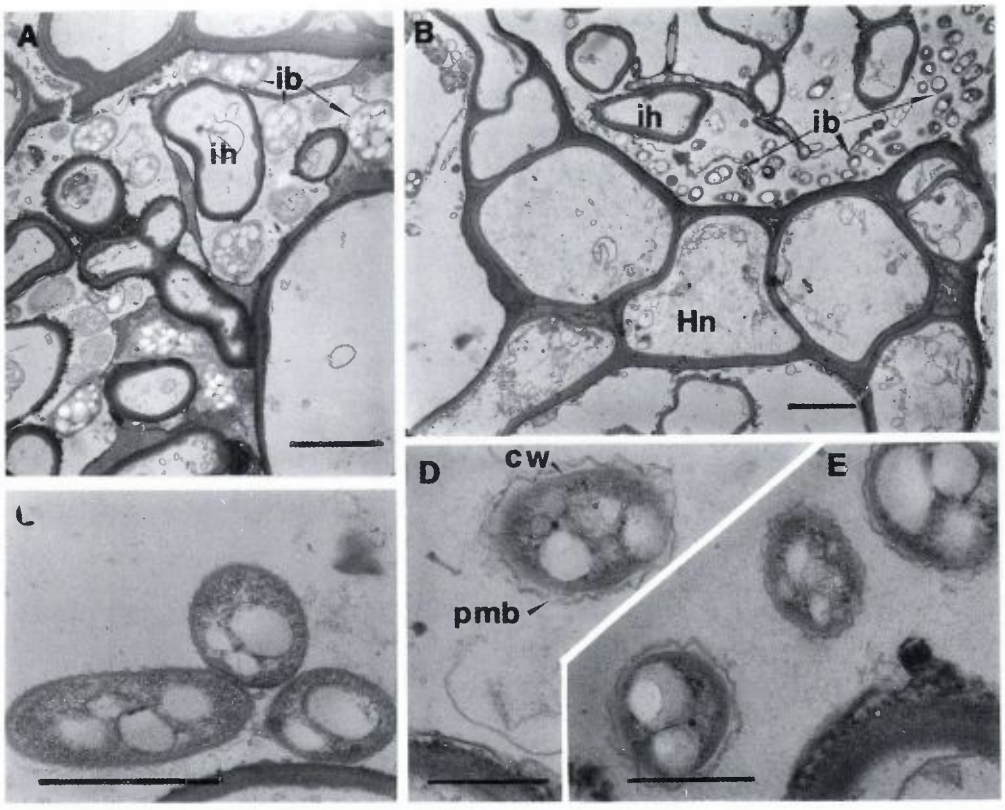

Figure 5. Transmission electron micrographs of sections of 12-month old arbutoid mycorrhizae showing invasion of root cells by bacteria (A, B), bacteria external to the hyphal mantle (C), details of the bacteria inside a root cell, with an outer envelope resembling a membrane (D, E): (ih) = intracellular hyphae; (ib) = bacteria within an epidermal root cell; (Hn) = Hartig net; (cw) = cell wall; (pmb) = plasmic membrane. Bars = 1μm.

the possible existence of an association between arbutoid mycorrhizae and *Azospirillum*-like bacteria.

Ultrastructural observations evidenced a wide homogeneity of the bacteria wherever they were located, around external hyphae, among and within the hyphae in the mantle and in plant cells invaded by the fungus. The uniformity of the bacterial colonies was maintained also in the pocket structures occurring frequently in the hyphal mantle: their organization appeared as gradually proceeding from the external to the internal part of the hyphal mantle, and

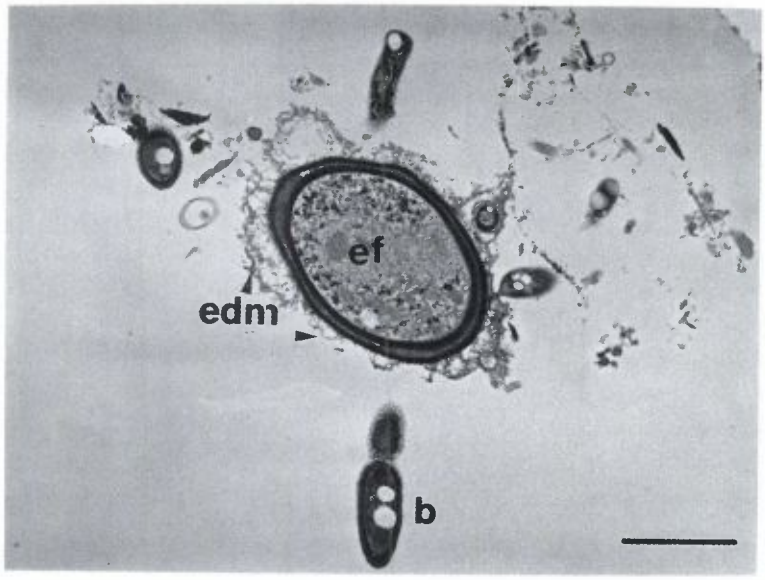

Figure 6. Transverse section of an external hypha of *A. unedo* mycorrhizal symbiont, showing bacteria around the hypha and attached to the electron-dense matrix enveloping the hypha: (ef) = external hypha; (edm) = electron-dense matrix; (b) = bacteria. Bar = 1μm.

from young to old mycorrhizae. In fact, in young mycorrhizae the microniches among the hyphae contained few bacteria immersed in an electron-dense matrix, in older mycorrhizae the bacteria increased in number to fill the entire niches. These findings are consistent with those recently obtained by Buscot (1994), who ultrastructurally evidenced the occurrence of an endobacterium in morel ectomycorrhizae.

It is possible that the quantity and quality of hyphal exudates or the production of extracellular material may operate as selecting agents toward the microorganisms; in fact, *Azospirillum*-like cells were found in high numbers only in the mycorrhizosphere and endosphere of arbutoid mycorrhizae, whereas they were absent from *Cenococcum*-type mycorrhizae and from non-mycorrhizal roots (Table 1) (Malajczuk, 1979).

The occurrence of these populations of bacteria, in addition to the mycorrhizal fungal symbionts, may affect the growth of *A. unedo*, and may be involved in the high resistance to adverse conditions of this species in the Mediterranean habitat, where it is endemic. In fact, *A. unedo* shows a high capability of surviving and recolonizing sites after fire, by means of resprouting

from buds in undergrounds again, so helping in the re-establishment of post-fire plant communities (Bellgard et al., 1994). Many speculations have tried to explain the occurrence of bacteria associated with mycorrhizal hyphae, and some authors have suggested the possible existence of complex associations, which could explain the fitness of some plant species in nature, despite the adverse environment (Ames and Bethlenfalvay, 1987; Buscot, 1994). Moreover, the frequent findings of N_2-fixing microorganisms in the mycorrhizosphere (Richards and Voigt, 1964; Li and Hung, 1987; Garbaye, 1991; Linderman, 1992) and within sporocarps of ectomycorrhizal fungi (Li and Castellano, 1987) suggest the possibility of a direct biological interaction between mycorrhizal fungi and associated microorganisms, which could be of great ecological significance for the fitness of mycorrhizal plant species.

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